

Affinity labelling of the G_{M2}-activator protein

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In contrast to ganglioside G_{M2} (G_{M2}), its fatty acid free derivative, lyso-G_{M2}, and *N*-acetyllyso-G_{M2} are hydrolyzed by purified human hexosaminidase A (Hex A). The addition of G_{M2}-activator stimulates the Hex A-catalyzed hydrolysis of G_{M2} and that of *N*-acetyllyso-G_{M2} much more than that of lyso-G_{M2}. Based on this observation *N*-bromoacetyllyso-G_{M2} was synthesized as a potential affinity ligand of the G_{M2}-activator protein. *N*-Bromo[³H]acetyllyso-G_{M2} inactivated the G_{M2}-activator selectively and irreversibly by covalent modification.

Ganglioside Hexosaminidase Lysoganglioside G_{M2} Bromoacetyllysoganglioside G_{M2}

1. INTRODUCTION

Two proteins, Hex A and the G_{M2}-activator protein, are involved in the lysosomal degradation of ganglioside G_{M2} [1]. Inherited deficiencies of each of these proteins result in fatal ganglioside storage disorders [2]. The G_{M2}-activator is an enzymatically inactive glycolipid-binding protein which stimulates the glycolipid transfer from donor to acceptor liposomes [3]. Binding studies indicate that it forms a water-soluble, stoichiometric complex with ganglioside G_{M2} which is the putative substrate of Hex A as suggested by kinetic analysis [4]. Here we present direct support for the complex formation

by affinity labelling of the G_{M2}-activator protein using an analogue of ganglioside G_{M2}, *N*-bromo[³H]acetyllyso-G_{M2}.

2. EXPERIMENTAL

2.1. Synthesis of [³H]lyso-G_{M2}, [³H]NAC-lyso-G_{M2} and [³H]NBrAc-lyso-G_{M2}

Ganglioside G_{M2} was deacylated and the amino group of its sphingosine moiety selectively blocked as described [5] using fluorenylmethoxycarbonyl chloride as protecting reagent in a two-phase system. Acetylation of the free amino group of the neuraminic acid residue with [³H]acetic anhydride (18 500 GBq/mol) followed by acetic anhydride and the removal of the protecting group in liquid ammonia yielded 30% [³H]lyso-G_{M2} (1100 GBq/mol). Acylation of [³H]lyso-G_{M2} with either acetic anhydride or bromoacetic anhydride in 5% NaHCO₃ solution at 0°C, followed by dialysis against water yielded 90% [³H]NAC-lyso-G_{M2} (1100 GBq/mol) and 90% [³H]NBrAc-lyso-G_{M2} (1100 GBq/mol), respectively.

2.2. Incubation with Hex A

The incubation volume of 40 µl contained 10 nmol glycolipid as substrate, 10 mM citrate buffer, pH 4.0, 60 nmol NaN₃, 10 µg bovine serum albumine, 220 mU Hex A [6] and, when indicated,

Abbreviations: G_{M2}, ganglioside G_{M2}, II³Neu5Ac-GgOse₃Cer, monosialogangliosylceramide; lyso-G_{M2}, II³Neu5Ac-GgOse₃Sph, monosialogangliosylsphingosine; G_{M3}, ganglioside G_{M3}, II³Neu5Ac-LacCer, monosialolactosylceramide; lyso-G_{M3}, II³Neu5AcLacSph, monosialolactosylsphingosine; NAc-lyso-G_{M2(3)}, *N*-acetyllyso-G_{M2(3)}; NBrAc-lyso-G_{M2(3)}, *N*-bromoacetyllyso-G_{M2(3)}; MUGlcNac, 4-methylumbelliferyl-β-D-*N*-acetylglucosaminid; MUGalNac-6-SO₄, 4-methylumbelliferyl-β-D-*N*-acetylgalactosaminid-6-sulfate; Hex A, hexosaminidase A, β-2-acetamido-2-desoxy-D-glucosid; acetamidodesoxyglucohydrolase, EC 3.2.1.30; *tlc*, thin-layer chromatography

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1 unit [4] of activator protein. After incubation at 37°C the mixtures were analyzed by TLC using silica gel 60 plates and chloroform:methanol:15 mM CaCl₂ (60:35:8, by vol.) as solvent system. Distribution of radioactivity on the dried plates was analyzed employing a radioscaner (Berthold LB 2723).

2.3. Preparation of GM₂-activator protein

GM₂-activator was prepared from postmortem human kidneys as described [4]. A crude preparation was obtained after extraction, heat- and acid precipitation, ion-exchange and gel chromatography. A purified preparation was obtained after fractionation by further steps: hydrophobic chromatography on octyl-Sepharose and HPLC (silica gel TSK G 2000 SW; solvent 100 mM phosphate buffer pH 6.9).

Proteins of the crude GM₂-activator preparation were radiolabelled: the protein mixture was dissolved in 5% NaHCO₃ solution and tritium-labelled by reaction with 2 μ mol [³H]acetic anhydride (18 500 GBq/mol in toluol, 3.7 GBq/ml) and then dialyzed against water.

2.4. Affinity labelling

GM₂-activator (purified or crude preparation, containing 2.5 nmol GM₂-activator each) was dissolved in 200 μ l of 20 mM citrate buffer, pH 4.0, 10 mM NaN₃. [³H]NBrAc-lyso-GM₂ (3 nmol, 1100 GBq/mol), dissolved in 6 μ l water, was added and the mixture incubated for 23 h at 37°C, dialyzed against water (20 h) and lyophilized. Aliquots of 20% were subjected to SDS electrophoresis in a 12.5% acrylamide gel according to Laemmli [7]. Radioactive bands were visualized by fluorography [8,9].

3. RESULTS AND DISCUSSION

In the absence of detergents or GM₂-activator purified Hex A has no detectable activity against the ganglioside GM₂ but hydrolyzes lyso-GM₂ and NAc-lyso-GM₂ (fig.1). Ganglioside GM₂ forms rather stable micelles in aqueous solution with a CMC at or below 10⁻⁹ M [10]. Ganglioside micelles are not recognized as substrate by Hex A [11]. Lyso derivatives have only one hydrophobic chain, the sphingosine moiety, and probably a much higher CMC. In this case monomers might

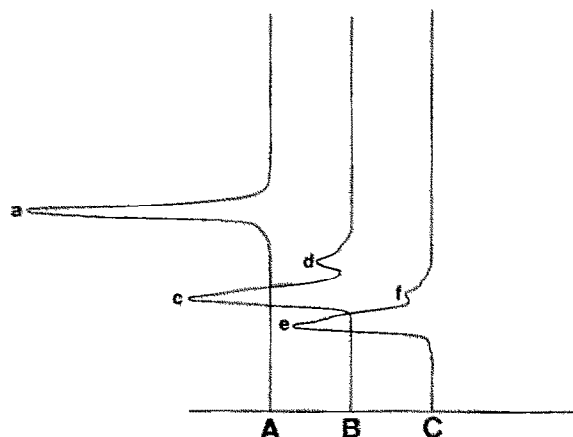


Fig.1. Hydrolysis of [³H]GM₂ (A), [³H]NAc-lyso-GM₂ (B) and [³H]lyso-GM₂ (C) by Hex A in the absence of GM₂-activator. Incubation mixtures were run for 10 h at 37°C and analyzed by TLC (as described in section 2). Distribution of radioactivity on the TLC plate was analyzed with the help of a radioscaner. (a) [³H]GM₃, (c) [³H]NAc-lyso-GM₂, (d) [³H]NAc-lyso-GM₃, (e) [³H]lyso-GM₂, (f) [³H]lyso-GM₃.

be available for interaction with Hex A. Addition of GM₂-activator to the incubation mixtures stimulates the degradation of GM₂ and NAc-lyso-GM₂ much stronger than that of lyso-GM₂ (fig.2). In the incubation mixture at pH 4 a positive charge on the free amino group of sphingosine

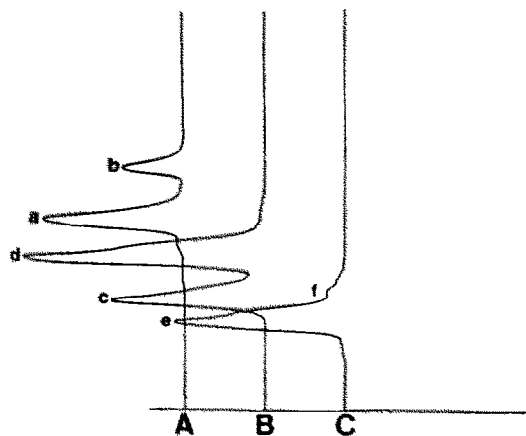


Fig.2. Hydrolysis of [³H]GM₂ (A), [³H]NAc-lyso-GM₂ (B), and [³H]lyso-GM₂ (C) by Hex A in the presence of GM₂-activator protein. The assay mixtures were incubated for 2 h at 37°C and analyzed by TLC and radioscaning as described in section 2. (a) [³H]GM₂, (b) [³H]GM₃, (c) [³H]NAc-lyso-GM₂, (d) [³H]NAc-lyso-GM₃, (e) [³H]lyso-GM₂, (f) [³H]lyso-GM₃.

presumably inhibits complex formation between the lyso- G_{M2} and the G_{M2} -activator protein. This suggests a positive group, such as lysine residue, in the lipid binding site of the G_{M2} -activator. Therefore an analogue of ganglioside G_{M2} , NBrAc-lyso- G_{M2} , was synthesized as a potential affinity ligand for labelling. This analogue is rather similar to NAc-lyso- G_{M2} and G_{M2} , contains as a reactive group a properly positioned bromoacetyl residue, which may react with a nucleophile of the protein binding region, but is rather inert to unspecific hydrolysis. In contrast to NAc-lyso- G_{M2} , NBrAc-lyso- G_{M2} inhibits irreversibly the degradation of [3H] G_{M2} by Hex A in the presence of G_{M2} -activator (not shown). On the other hand, it inhibits less than NAc-lyso- G_{M2} the hydrolysis of MUGa1NAc-6-SO₄ and MUG1cNAC by either Hex A or Hex B (not shown). Incubation

of [3H]NBrAc-lyso- G_{M2} with either pure or crude G_{M2} -activator preparations resulted in radioactive labelling of the activator protein (fig.3). Under the conditions used the G_{M2} -activator was specifically labelled among all the proteins present in the crude activator preparation (fig.3). If, however, the crude G_{M2} -activator preparation is incubated with increasing amounts of [3H]NBrAc-lyso- G_{M2} other protein bands are also labelled. In fig.3 the G_{M2} -activator protein shows up as a double band like the purified activator protein [12]. This is probably due to the heterogeneity of its carbohydrate portion (unpublished). The affinity labelling, shown in fig.3 directly supports the postulated ganglioside G_{M2} - G_{M2} -activator protein complex [4]. The derivatized activator protein should be helpful in the elucidation of its lipid-binding site.

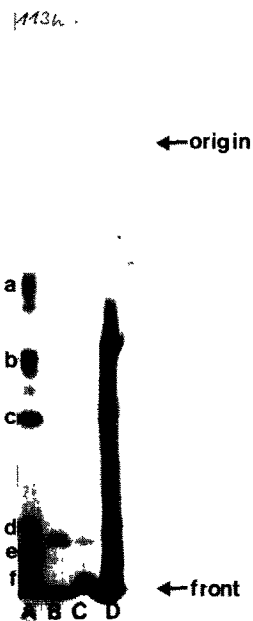


Fig.3. SDS gel electrophoresis of G_{M2} -activator preparations after affinity labelling (as described in section 2). The exposure time of the fluorogram was 113 h. Lanes: (A) standards: (a) bovine serum albumin (69 kDa), (b) ovalbumin (46 kDa), (c) carbonic anhydrase (30 kDa), (d) soybean trypsin inhibitor (21.5 kDa), (e) lactoglobulin A (18.37 kDa), (f) cytochrome c (12.3 kDa). (B) Purified G_{M2} -activator protein which yields a double band after incubation with [3H]NBrAc-lyso- G_{M2} , (C) crude G_{M2} -activator preparation after incubation with [3H]NBrAc-lyso- G_{M2} , (D) labelling of all protein bands present in the crude G_{M2} -activator preparation by [3H]acetic anhydride.

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